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AN IMPROVED SONICATION METHOD FOR REMOVAL OF MICROORGANISMS FROM SURFACES

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ABSTRACT

This report describes approaches, efforts, and results to improve the technique of removing microorganisms from stainless steel strip surfaces by use of ultrasonic cavitation. Recommendations are made to enable others performing these operations to effect a more efficient removal in less time.

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I. INTRODUCTION

With the advent of the Planetary Quarantine activities, the requirements for determining the number of viable organisms in a given environment has become more stringent. Greater numbers of samples must be taken, and greater accuracy in the assaying of the samples is required. The use of 1" x 2" sampling strips has become a commonly used method for collecting particles for analysis. These strips are first cleaned and sterilized, then used to collect particles by airborne fallout in a given area or to collect particles by impaction in laminar flow devices. After the particles have been collected on the strips, they must be removed from these strips and suspended in a liquid for subsequent processing to determine the number of viable particles. This removal has been and is being accomplished by use of ultrasonics. After using the recommended method of removal by sonication, ideas for improving the method were formulated, and efforts were initiated to optimize the removal operation. The improved method utilizes the same ultrasonic equipment, but a more repeatable removal with a higher percentage of removal in a shorter time is achieved.

Present Practices

The standard procedure for removal by sonication* states that "the ultrasonic bath shall conform to the following specifications:

1. The frequency shall be 25 kc/sec.
2. The power output in relation to the bottom surface of the tank shall be at least 2.3 w/sq. in. (0.35 w/cm^2).

*As quoted from "STANDARD PROCEDURES FOR THE EXAMINATION OF SPACE HARDWARE." (NPQ-1, NASA, Washington, D. C.)

3. If the ultrasonic bath is not automatically tuned, tuning shall be performed according to the manufacturer's directions.
4. The inside surfaces of the bath shall be stainless steel.
5. Glass bottles containing piece-parts or stainless steel strips shall be supported on the bottom of the tank.
6. The tank fluid shall be an aqueous solution of 0.3% by volume polyoxyethylene sorbitan mono-oleate (Tween 80).
7. The temperature of the bath fluid shall be at least 25c and shall not exceed 37c. The bath fluid shall be changed periodically in order to prevent heat-up.
8. The bath liquid shall be at least one inch above the level of the liquids in the bottles being ultrasonicated."

The standard procedures designate the container for the strips during the time of ultrasonication to be wide mouth, screw-capped, square 4-oz. bottles (Bussey Products Company, 2750 West 35th Street, Chicago 32, Illinois, Cat. No. 1463 or equivalent).

The other pertinent procedure as per the Standard Procedures is to "add 50ml, 1.0% peptone water maintained at 25±5c to each bottle containing a stainless steel strip. Place the bottles containing 1.0% peptone water and the stainless steel strips to be assayed in an ultrasonic bath and ultrasonicate for 12 minutes."

II. ANALYSIS OF PROBLEMS

The standard method has four characteristics which allowed possible improvements. These are: (1) A more repeatable method, (2) a higher percentage of removal, (3) a shorter time of ultrasonication, (4) utilization of more water in the ultrasonic bath to reduce the temperature rise in the ultrasonic tank. Additionally, it was noted that with 50ml of liquid in the bottle there is a small segment of the 1" x 2" strip that is not submerged,negating the possibility of removal from this area. Figure 1 shows the Bussey bottle and the strips. As the 1" x 2" strip rests in the bottle, the strip is approximately 40^{o} from horizontal. In this position the radiated energy from the transducer at the bottom of the tank sees a thicker part than it would if the strip were horizontal, and the adsorption of energy by the strip is lessened. This causes greater loss by attenuation than is optimum. The bottom of the bottle is irregularly shaped but is generally concave. This is undesirable for two reasons: First, this shape is much more conducive to the formation of air bubbles on the bottom exterior of the bottle which would attenuate the energy down to zero. The second reason is that this concave surface causes unwanted reflections of the energy waves. These reflections may cause the energies to be focused inside the bottle resulting in a higher cavitation intensity in a small localized area. However, it also leaves many areas inside the bottle with somewhat lower cavitation intensity. Also, the glass is thicker at the periphery on the bottom of the bottle causing attenuation of the ultrasonic energy.

The water level aspect appeared to be no major problem since support racks can be fabricated to allow the water level to be raised to approximately 0.75 inch below the top of the tank top. Throughout the tests covered in this report, the ultrasonic cleaner was operated for two minutes

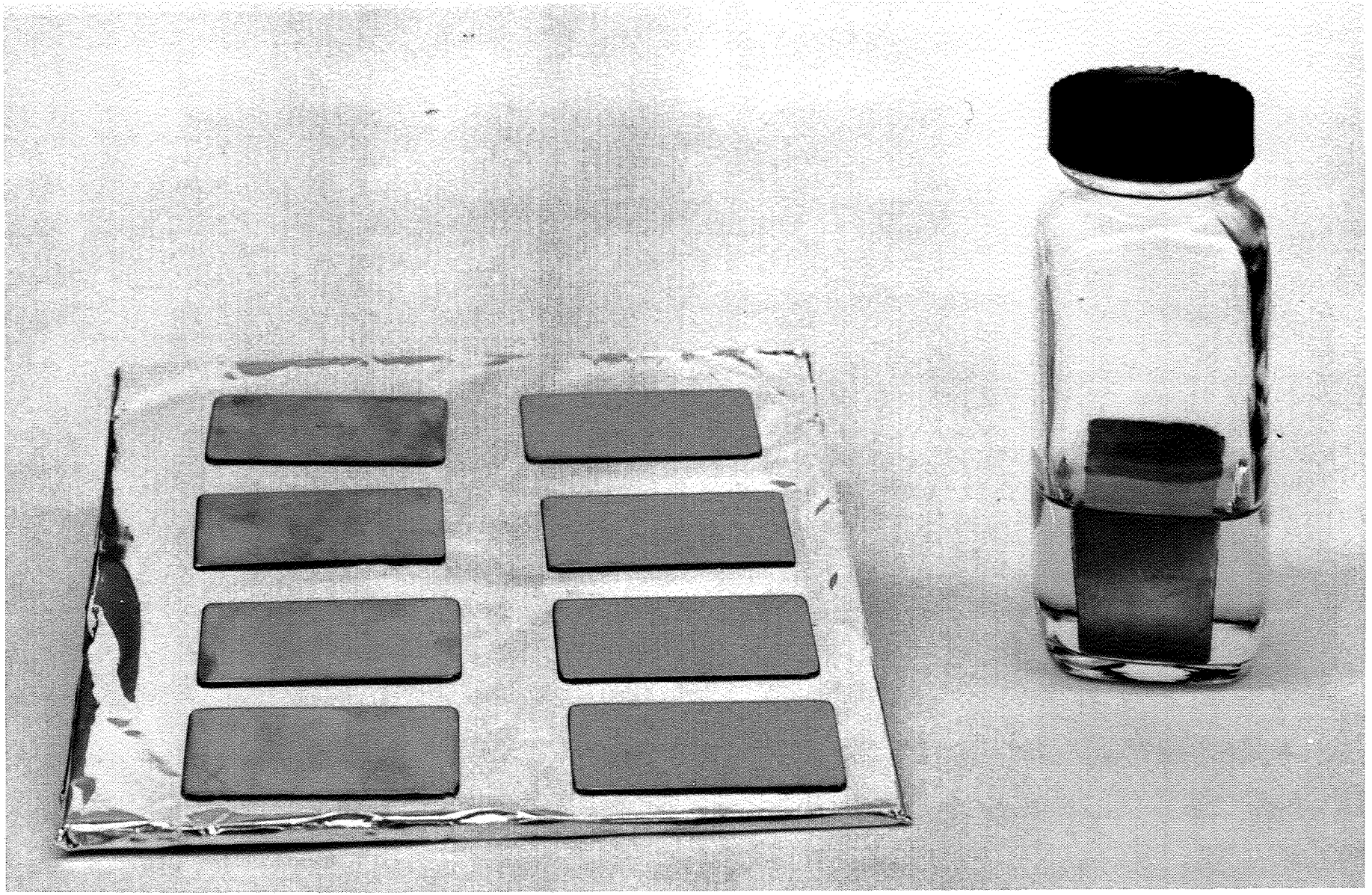

FIGURE 1 - Bussey bottle and stainless steel strips

prior to start of the day's operation for degassing purposes. This degassing tends to produce stability of the cavitation intensity.

III. TESTS AND DATA

The ultrasonic equipment used in the test described herein was neither the same model nor make as defined in Appendix C.4 of the "Standard Procedures for the Microbiological Examination of Space Hardware." However, the equipment did meet or exceed the specifications in all respects. The tests were all conducted in a comparison mode so that all the equipment was the same on all tests. Figure 2 shows the ultrasonic equipment used in this testing.

The stainless steel strips which were used in the test procedures were innoculated with spores in their individual states in an ethanol suspension since this represented a difficult situation for removal. The spores used in these experiments were Bacillus subtillis var. niger in 95% ethanol, approximately 8×10^8/ml. Dilutions to 8×10^3/ml were made in ethanol. Aliquots of 0.1 ml. of the final dilution were innoculated onto the stainless steel strips with a sterile 1.0 ml. pipette. The innoculated strips were allowed to dry in open sterile petri dishes under a laminar downflow clean bench. Then the dishes were covered immediately.

The processing and counting of the samples was performed according to "Standard Procedures for the Microbiological Examination of Space Hardware." The single exception to this was that each strip was rinsed with approximately 10 ml. of sterile peptone water after it was sonicated and before it was overlaid with Trypticase Soy Agar. This was done so that the assay of the bacteria on the strip would include only those firmly attached and not those present in the contaminated fluid wetting the strip's surface upon removing it from the sonication bath. The data in the tables will not show the individual aliquot reading, but will be summarized as "off" count (organisms removed), "on" count (organisms left on), and percentage of removal.

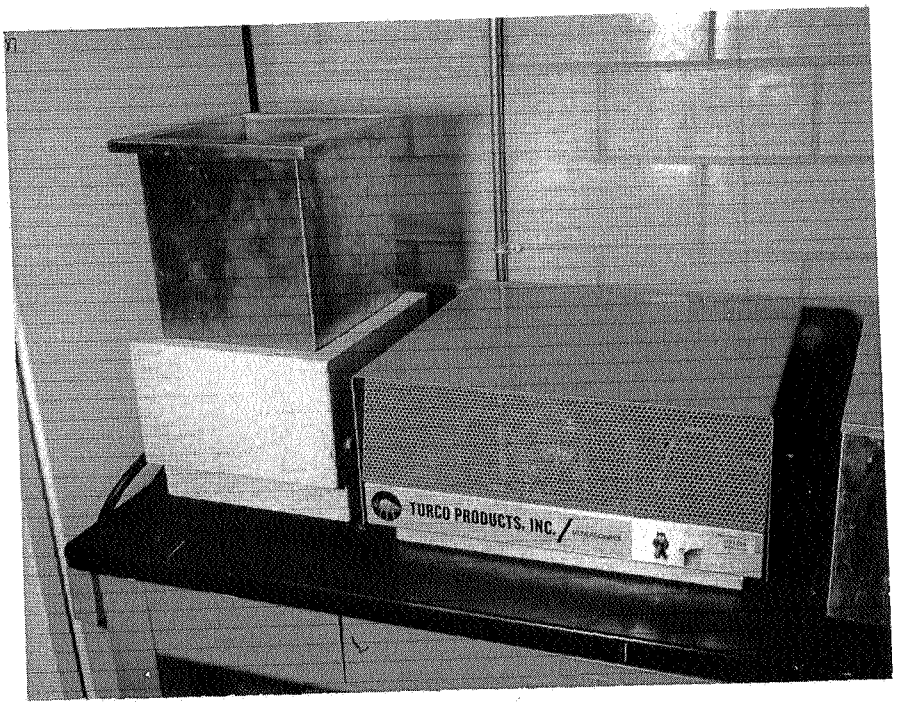

FIGURE 2 - Ultrasonic Cleaner

In the early tests the recommended NASA Method was used as a comparison to the experiment being performed. All tests employing the Bussey bottles were ultrasonicated for 12 minutes. After evaluating the problem of removing the microorganisms, five minutes was arbitrarily set as the maximum time required to effect desired removal. Therefore, on tests utilizing containers other than the Bussey bottles, the time of sonication was limited to five minutes unless otherwise specified. The initial tests were set up to establish that the ultrasonic cavitation is the contributing factor to the removal (as compared to solvent action) and that a different container and positions delivered improvements. Table 1 shows the results of tests using eight strips with two each being hand agitated in Bussey bottles, Bussey bottles at the bottom of the tank (NASA Method), Bussey bottles near the top of the tank, and 250 ml. beakers near the top of the tank. The strips lay flat in the bottom of the beaker. The orientation of the innoculated side of the strip with respect to the bottom of the beaker is noted in the data tables. Table 2 shows the data from a test identical to test #1. Figure 3 shows the 250 ml. beaker.

Table 3 shows the results from a test where, in addition to the experiments in Tables 1 and 2, a mechanical agitator, Arthur H. Thomas Model 8917A, was used, and two each 250 ml. beakers were used at the bottom of the tank. From the tests this far, it has been found that the ultrasonic energy is the contributing factor to the removal and that solvent action is minimal. No further tests were conducted with hand agitation or mechanical agitation. From the data thus far, it was concluded that the analysis of the removal problem was correct and that the beakers were doing a more effective job of removal.

FIGURE 3 - 200 ml. Beaker

TABLE 1

Method	OFF COUNT	ON COUNT	% REMOVAL
Hand Agitated	0	221	0%
Hand Agitated	15	214	06.6%
Bussey Bottle Bottom	550	4	99%
Bussey Bottle Bottom	360	6	98%
Bussey Bottle Top	200	114	64%
Bussey Bottle Top	260	45	85%
Beaker Top (Down)	510	3	99%
Beaker Top (Up)	620	1	99.9%

TABLE 2

Method	OFF COUNT	ON COUNT	% REMOVAL
Hand Agitated	15	248	05.7%
Hand Agitated	0	286	0.0%
Bussey Bottle Bottom	230	148	61%
Bussey Bottle Bottom	155	142	52%
Bussey Bottle Top	585	82	88%
Bussey Bottle Top	120	105	53%
Beaker Top (Up)	260	36	88%
Beaker Top (Down)	465	12	97%

TABLE 3

Method	OFF COUNT	ON COUNT	% REMOVAL
Hand Agitated	15	700	2%
Hand Agitated	11	744	1%
Mechanical Agitated	110	640	15%
Mechanical Agitated	75	504	13%
Bussey Bottle Top	570	261	69%
Bussey Bottle Top	160	744	20%
Bussey Bottle Bottom	100	708	12%
Bussey Bottle Bottom	80	990	7%
Beaker Top (Down)	1055	0	100%
Beaker Top (Up)	985	26	97%
Beaker Bottom (Up)	980	15	99%
Beaker Bottom (Down)	1025	26	98%

The Sandia Laboratory glass shop fabricated special containers of 2mm thick glass. These containers were cylindrical in shape with an inside diameter of 7 cm. and a height of 8.5 cm. At a level 5 cm up from the bottom there are three each glass protrusions, 2 mm in their outside diameter, extending outward 6 mm, and spaced 120^{0} from each other on the outer periphery of the container. These serve to support the container in a rack near the top of the ultrasonic tank. Figure 4 shows the new containers. Table 4 shows the results from tests of three each Bussey Bottles in the NASA approved method, three each 250 ml. beakers, and three each of the new beakers. On the test shown in Table 4 the contaminants were intramural organisms and were "settled out" from a dusty enclosure instead of being pipetted on. Table 5 shows the results of a test series identical to those in Table 4 except that B. subtillis var. niger was used and was applied via the pipette method. Table 6 is a table showing the results from a test series identical to that in Table 5. This test was the final test to verify that the project had progressed to a nearly optimum configuration of container.

The next logical step was to ascertain the time requirements. Table 7 shows the results from the initial sonication time profile test. Since it had been established from previous tests that with the new containers five minutes was sufficient time, three samples each were sonicated at 1, 2, 3, 4 and 5 minutes. There were only 12 of the new containers on hand, and this test series required 15, so the standard 250 ml. beakers were used on the one minute group. The rack for these containers was arranged so that five beakers could be processed simultaneously (one in the center and one in each of the four corners).

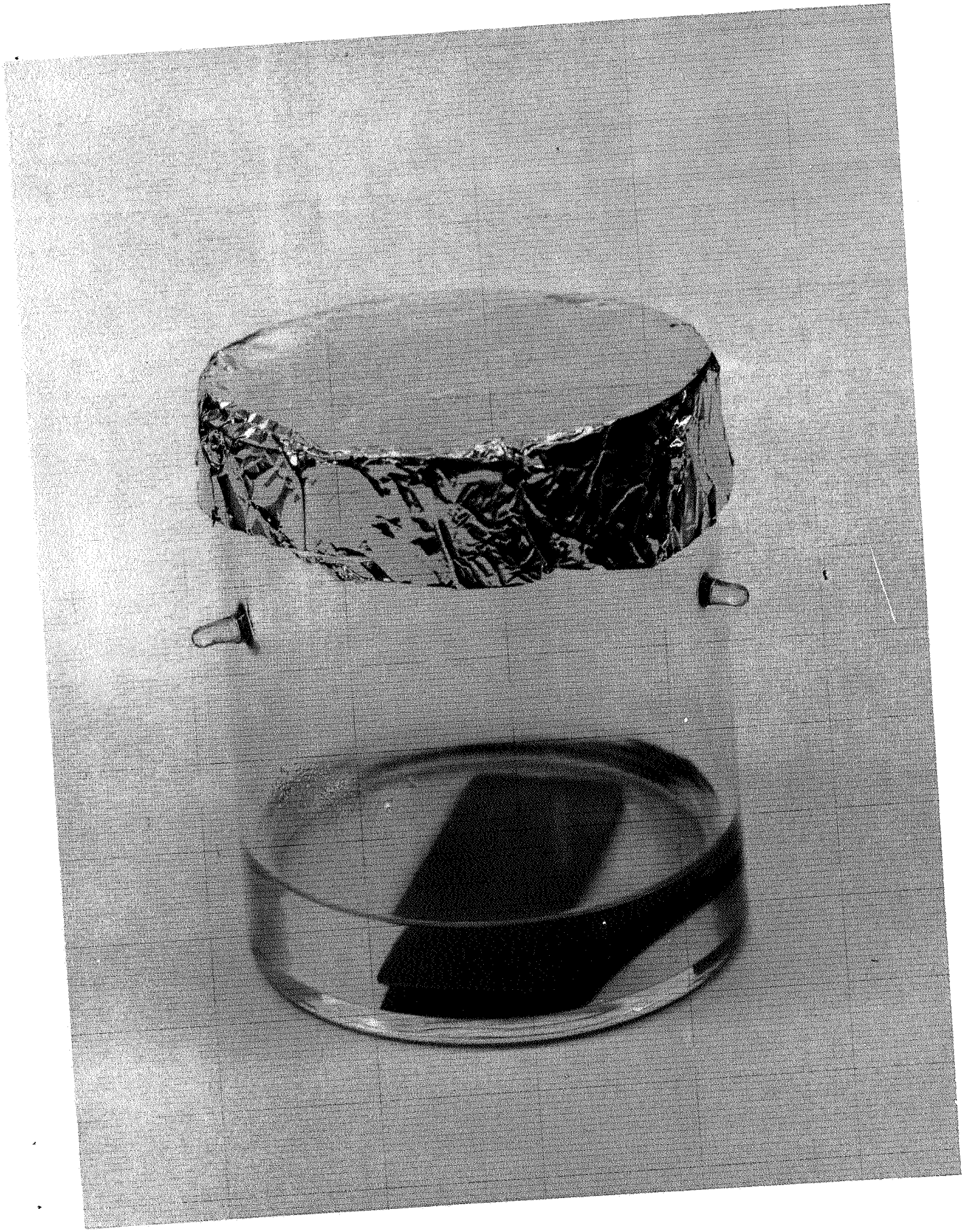

FIGURE 4 - New Beaker

TABLE 4

Method	OFF COUNT	ON COUNT	% REMOVAL
Bussey	3	3	50%
Bussey	7	2	80%
Bussey	7	1	88%
250 ml BKR	211	1	99+%
250 ml BKR	86	0	100%
250 ml BKR	42	0	100%
New BKR	8	0	100%
New BKR	6	0	100%
New BKR	3	0	100%

TABLE 5

Method	OFF COUNT	ON COUNT	% REMOVAL
Bussey	877	19	98%
Bussey	1200	39	97%
Bussey	1170	41	97%
250 ml BKR	1077	16	99%
250 ml BKR	1230	0	100%
250 ml BKR	1520	0	100%
New BKR	1150	4	99+%
New BKR	1350	0	100%
New BKR	1327	0	100%

TABLE 6

Method	OFF COUNT	ON COUNT	% REMOVAL
Bussey	800	157	84%
Bussey	710	192	79%
Bussey	440	249	70%
250 ml BKR	1935	247	89%
250 ml BKR	1130	3	99+%
250 ml BKR	240	179	57%
New BKR	1190	8	99+%
New BKR	1050	46	96%
New BKR	1195	15	99%

TABLE 7

Time (Min.)	OFF COUNT	ON COUNT	% REMOVAL
1 CP	TNTC	3	99+%
1	3105	36	99%
1	2770	46	99%
2	2820	7	99+%
2 CP	2810	2	99+%
2	2230	29	99%
3	2555	113	96%
3 CP	2735	28	99%
3	2235	87	96%
4	2540	36	99%
4 CP	3025	2	99+%
4	2350	68	98%
5	2655	23	99%
5 CP	3125	2	99+%
5	2510	48	98%

CP: Center position in ultrasonic tank

Three beakers were processed each time and were arranged diagonally across the tank. The previous tests were conducted with each beaker being sonicated individually. The beaker in the center position was so marked, and the data reflects the difference in removal of the center position as compared to that on the beakers in the outer extremities. Table 8 shows the results from another time sonication profile test. On this test the standard 250 ml beakers were used on the 5 minute test. Table 9 shows the results of the final time test. Only one and two minutes were used since previous tests had sufficed for the longer times.

Table 10 shows the data from a test series to determine the reliability of the method which previous tests found to be optimum. This new recommended method is described later in this report. In order to preclude any baising effects from human factors, different personnel were used on this series from the other tests. As can be seen from the table the removal percentage only varies from 99.6% to 99.9%.

Two additional statements can be made concerning findings in the course of the experimentation. The data shows good repeatability and efficiency for different loading levels. Also, known concentrations of Escherichia coli and Serratia marcescens were sonicated for periods up to 12 minutes to determine if they were harmed by the assay procedure. No noticeable decay was found in their populations following the ultrasonic treatment.

TABLE 8

Time (Min.)	OFF COUNT	ON COUNT	% REMOVAL
1	850	134	86.4%
1 CP	1415	0	100%
1	1400	36	97.6%
2	1025	32	97.2%
2 CP	1280	1	99.9%
2	50	377	11.7%
3	90	270	25%
3 CP	1055	5	99.5%
3	370	180	67.3%
4	1490	9	99.4%
4 CP	1560	1	99.9%
4	1685	21	98.8%
5	1285	3	99.9%
5 CP	1130	1	99.9%

CP: Center position in ultrasonic tank

TABLE 9

Time (Min.)	OFF COUNT	ON COUNT	% REMOVAL
1	920	207	81.7%
1 CP	1705	18	99%
1	445	1	99.9%
2	1010	31	97%
2 CP	1330	0	100%
2	705	107	86.8%

CP: Center position in ultrasonic tank

TABLE 10

Time (Min.)	OFF COUNT	ON COUNT	% REMOVAL
New BKR	11440	22	99.8%
2	8610	10	99.9%
	11120	19	99.8%
	10800	6	99.9%
	8770	31	99.6%
	9830	8	99.9%
	10390	15	99.9%
	10320	21	99.8%
	9670	2	99.9%

IV. RECOMMENDATIONS

Based on the data obtained from tests as previously described, an improved method of removal of microorganisms from surfaces by ultrasonication is easily achievable. The results of the tests show that one minute of ultrasonication under optimized conditions gives an extremely efficient removal percentage. However, for an added assurance of efficiency, it is recommended that the time of sonication be set at 2 minutes using the new configuration of containers, i.e., the 7 cm. ID beaker. This container should be supported from a rack at the top of the ultrasonic tank so that the liquid level of the tank will be approximately 1" below the top lip of the tank. This will cause the level of the bath and the level of the 50 ml. peptone water in the beaker to be nearly equal. This added water will preclude the rapid heat rise in the tank liquid as has been experienced in the past with the bottles set on the tank bottom. Also, using the full tank of water does not affect the temperature of the sonication medium as much. Since instability of removal efficiency was noted on the beakers near the tank walls, it is recommended that only one beaker be sonicated at a time and that this be placed at the center of the tank surface. The NASA specifications designates that 15 strips be processed within 60 minutes. This can be easily achieved by ultrasonicating each strip individually for two minutes. This consumes only 30 minutes and actually makes a smoother flow of operation since there are no "bottlenecks" or "buildups" in the processing line. It is also recommended that the strips be marked on one side and that the surface upon which the contaminants lay be placed down (facing the bottom) in the containers.

V. CONCLUSIONS

The work covered by this report has demonstrated three significant items concerning the removal of microorganisms from surfaces by use of ultrasonics. First, the verification that the ultrasonic cavitation is the prime contributor to the removal of these organisms and that the solvent action is minimal has confirmed that those who initiated the utilization of ultrasonics in the area of microbiology did, in fact, make a substantial advancement to the state-of-the-art. Secondly, it has been found that with minor refinements this removal can be made a highly efficient, reliable, and repeatable operation. And thirdly, that this removal can be accomplished in two minutes as compared to twelve minutes previously required has been demonstrated.

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